REMARKS

Claims 7-16 are pending and claims 9, and 13 to 16 are withdrawn from consideration. Claim 7 has been amended to emphasize that claim 7 corresponds substantially to claims 1-19 of United States Patent No. 6,033,886, issued March 7, 2000, cited as reference BT in the Supplemental Information Disclosure Statement filed March 22, 2001 (see also the Statement Under 37 C.F.R. § 1.607(c) filed July 9, 2003). New claims 17 to 21 have been added. Support for the new claims can be found in the specification as originally filed, *e.g.*, as set forth in the chart below. No new matter has been introduced. Thus, claims 7 to 21 will be pending upon entry of the present amendment.

Claim	Support
7	Page 6, line 37 to page 7, line 1; Page 43, lines 3-6; page 36, line 30; page 22, lines 8-21
17	Page 27, lines 1-25
18	Page 28, lines 10-13
19	Page 21, lines 31-36; page 26, lines 1-11; Table II starting at page 62; page 33, lines 1-2; page 36, lines 33-37
20	Page 1, line 12
21	Page 45, line 33 to page 48, line 24

The Rejection under 35 U.S.C. § 112, First Paragraph, Should Be Withdrawn

Claim 12 was rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. In particular, the Examiner contends that Applicants have not demonstrated that any of the modified RSV disclosed in the application would be effective as vaccine against RSV infections. Applicants respectfully disagree because the specification as filed provides sufficient teachings to enable the skilled artisan to make and use the claimed invention.

Applicants submit concurrently herewith a publication that was co-authored by the inventor Hong Jin (Cheng *et al.*, 2001, Virology 283:59-68; "Cheng;" attached as Exhibit 1). Cheng shows that a recombinant RSV lacking the M2-2 open reading frame (rA2ΔM2-2) is attenuated in African green monkey and provides protection against infection with the wild-type virus (see Cheng, at page 63, Table 2 and page 64, Table 3).

The Examiner contends that claim 12 is directed to vaccines against any non-segmented, negative-stranded RNA virus although the presently claimed invention is focused on vaccines against RSV. Applicants respectfully point out that, in response to the Restriction Requirement of June 17, 2003, the invention of Group I was elected wherein RSV is the recombinant virus. As claim 7 is a linking claim, claim 7 must be examined with the invention elected, *i.e.*, RSV. See M.P.E.P. § 809.

THE LEGAL STANDARD

The test for enablement is whether one reasonably skilled in the art could make or use the invention, without undue experimentation, from the disclosure in the patent specification coupled with information known in the art at the time the patent application was filed. U.S. v. Telectronics Inc., 857 F.2d 778, 8 USPQ2d 1217 (Fed. Cir. 1988). In fact, well known subject matter is preferably omitted. See Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1384 (Fed. Cir. 1986) ("a patent need not teach, and preferably omits, what is well know in the art."). Further, one skilled in the art is presumed to use the information available to him in attempting to make or use the claimed invention. See Northern Telecom, Inc. v. Datapoint Corp., 908 F.2d 931, 941 (Fed. Cir. 1990) ("A decision on the issue of enablement requires determination of whether a person skilled in the pertinent art, using the knowledge available to such a person and the disclosure in the patent document, could make and use the invention without undue experimentation."). These enablement rules preclude the need for the patent applicant to "set forth every minute detail regarding the invention." Phillips Petroleum Co. v. United States Steel Corp., 673 F. Supp. 1278, 1291 (D. Del. 1991); see also DeGeorge v. Bernier, 768 F.2d 1318, 1323 (Fed. Cir. 1985).

Undue experimentation is experimentation that would require a level of ingenuity beyond what is expected from one of ordinary skill in the field. Fields v. Conover, 170 USPQ 276, 279 (CCPA 1971). The factors that can be considered in determining whether an amount of experimentation is undue have been listed in In re Wands, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Among these factors are: the amount of effort involved, the guidance provided by the specification, the presence of working examples, the amount of pertinent literature and the level of skill in the art. The test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, so long as it is merely routine. *Id.*

Further, while the predictability of the art can be considered in determining whether an amount of experimentation is undue, mere unpredictability of the result of an experiment is not a consideration. Indeed, the Court of Custom and Patent Appeals has specifically cautioned that the unpredictability of the result of an experiment is not a basis to conclude that the amount of experimentation is undue in In re Angstadt, 190 USPQ 214 (CCPA 1976):

> [If to fulfill the requirements of 112, first paragraph, an applicant's] disclosure must provide guidance which will enable one skilled in the art to determine, with reasonable certainty before performing the reaction whether the claimed product will be obtained, ... then all "experimentation" is "undue" since the term "experimentation" implies that the success of the particular activity is uncertain. Such a proposition is contrary to the basic policy of the Patent Act. *Id.* at 219.

THE INSTANT SPECIFICATION PROVIDES AMPLE GUIDANCE TO THE SKILLED ARTISAN FOR MAKING AND USING THE CLAIMED CHIMERIC VIRUSES AS VACCINES

The instant specification, together with information which was readily available to the skilled artisan at the time the instant application was filed, provides a disclosure which fully enables the claimed invention.

Applicants respectfully point out that the specification as originally filed provides ample guidance for how to make and use vaccines comprising a genetically manipulated, infectious virus. At page 26, line 20 to page 30, line 5 of the specification as filed, approaches for how to prepare the vaccine formulations of the invention are described. Further Applicants respectfully point out that the skill in the field of Molecular Biology is very high. Thus, the skilled artisan would have been able at the time of filing of the application to generate the claimed vaccine formulations.

Further, the Examiner contends that the Applicants have not provided any examples of a modified RSV that is suitable as a vaccine. Applicants respectfully disagree. Section 10.1 of the specification as filed (page 59, line 35 to page 61, line 17) provides for the generation of M2-2 deletion mutants of RSV. As demonstrated by Cheng (Exhibit 1) such recombinant RSV M2-2 deletion mutants are suitable as vaccines. The recombinant RSV M2-2 deletion mutants described in Cheng were prepared as described in the instant specification, *e.g.*, at page 59, line 35 to page 61, line 17. Cheng provides evidence that the

recombinant RSV M2-2 deletion mutants provide partial protection against wild-type RSV in African Green Monkeys (see, *e.g.*, Cheng at page 62, left column, lines 10-13).

Applicants respectfully direct the Examiner's attention to section 2164.02 of the MPEP:

> "[...] if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate."

As African Green Monkeys are an accepted model system in the art for testing vaccines, the data provided in Cheng are sufficient to demonstrate that the presently claimed viruses are suitable as vaccines.

Further, Applicants respectfully point out that procedures for testing a vaccine are routine in the art, and that the skilled artisan would be able to determine without undue experimentation which of the recombinant viruses covered by the pending claims confer immunity to a subject when administered as a vaccine.

Thus, screening procedures to test recombinant viruses of the invention for their potential to serve as vaccines and to confer immunity to a particular pathogen to a subject should not be considered undue experimentation since such procedures are well-known to the skilled artisan.

The Examiner points to Murphy *et al.*, 1994, Virus Research 32, 13-36 ("Murphy, 1994"); Kahn, 2000, Current Opinion in Pediatrics 12:257-262 ("Kahn") and Crowe, 2002, Vaccine 20: S32-S37 ("Crowe, 2002") to support the position that the development of safe and effective RSV vaccines is still problematic.

One of the obstacles presented in the references cited by the Examiner relates to the administration of formalin-inactivated RSV vaccines. Applicants respectfully submit that the present vaccines are not formalin-inactivated, but the attenuation of the viruses of the present invention is achieved by genetically modifying the genome of the virus. Further, the references cited by the Examiner discuss the use of subunit vaccines. One of the problems associated with such subunit vaccines is their poor immunogenicity (see Murphy, 1994 page 17). In contrast to the such subunit vaccines, the vaccines of the present invention comprise packaged virions, which maintain the immunogenic properties of the wild type virus. Further, Kahn describes the use of recombinant RSV for the generation of vaccines as the "most significant advance in the development of an RSV vaccine . . ." (Kahn, page 260). In

the section discussing recombinant RSV, Kahn does not describe obstacles, but on the contrary gives a positive outlook on the success of vaccines based on recombinant RSV. Likewise, Crowe 2002 describes the use of live vaccines derived via genetic engineering in a very positive light.

Applicants respectfully request that the rejections under 35 U.S.C. § 112, first paragraphs, of claim 12 be withdrawn.

The Rejections under 35 U.S.C. § 102(b) Should Be Withdrawn

Claims 7, 10, and 11 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Gharpure *et al.*, 1969, J. Virol. 3(4):414-421 ("Gharpure"). In particular, the Examiner contends that Gharpure teaches genetically manipulated RSV and therefore anticipates claim 7, 10, and 11. Applicants respectfully disagree, because Gharpure does not describe RS viruses in which deletions or insertions have been introduced into the genome of the virus. Rather, the RS mutants described in Gharpure are the result of single point mutations introduced by exposure of the virus to chemical mutagens. Claims 7, 10, and 11 recite that the modification be an insertion or a deletion, Claim 18 recites that the modification be an insertion, deletion or substitution of an entire viral open reading frame, Claim 19 recites that the modification be an insertion, deletion, or substitution of at least 15 to 30 nucleotides of the viral genome.

The standard for an anticipatory reference is set forth in *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987): "[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *See also Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236 (Fed. Cir. 1989)(holding that "[t]he identical invention must be shown in as complete detail as is contained in the . . . claim"). Further, a prior art reference must be an *enabling* reference to anticipate. See *Akzo N.V. v. U.S. Int'l Trade Comm'n*, 808 F.2d 1471, 1479 (Fed. Cir. 1986) ("the prior art reference must be enabling, thus placing the allegedly disclosed matter in the possession of the public."). *See also* MPEP § 2121.01; *In re Hoeksema*, 399 F.2d 269 (CCPA 1968) ("In determining that quantum of prior art disclosure which is necessary to declare an applicant's invention 'not novel' or 'anticipated' within section 102, the stated test is whether a reference contains an 'enabling disclosure'.").

Gharpure describes temperature sensitive (t.s.) mutants of RSV generated by exposure of the virus to mutagens, such as 5-FU and NTG, to result in point mutations in the genome of RSV. Gharpure determines that the frequency of resulting t.s. mutants and the low concentration of mutagens used is consistent with the generation of single nucleotide mutants (Gharpure at p. 419, col. 1). The stability of the mutants generated as determined by the reversion rate indicates that the t.s. phenotype is the result of one or more point mutations introduced into the viral genome. Gharpure does not teach nor suggest that mutations other than single point mutations, such as insertions or deletions, can be introduced into the genome of the virus. Because Gharpure does not teach all the elements of the claimed invention, it cannot anticipate the claimed invention. Hence, the rejection over Gharpure should be withdrawn.

Claims 7, 10, and 11 were rejected under 35 U.S.C. § 1.102(b) as allegedly being anticipated by Park *et al.*, 1991, Proc Natl Acad Sci 88:5537-5541 ("Park"). In particular, the Examiner contends that Park teaches genetically manipulated RSV and therefore anticipates claim 7, 10, and 11. Applicants respectfully disagree because Park does not teach each and every element of the claim.

Park describes the construction of a construct containing the open reading frame of the chloramphenicol acetyltransfease (CAT) gene flanked by the 5' and 3' terminal sequences of the Sendai virus genome (pSend-CAT construct; see Park, Figure 1). In contrast to the presently claimed viruses, however, the pSend-CAT construct does not encode any viral gene products, including those required to render the virus replication competent. Thus, Park can not anticipate the replication competent viruses of the claimed invention.

Thus, the rejection of claims 7, 10, and 11 of the present application over Park under 35 U.S.C. § 102(b) should be withdrawn.

Claims 7, 8, 10, and 11 are rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Collins *et al.*, 1993, Virology 195:252-256 ("Collins I") or by Collins *et al.*, 1991, PNAS 88:9663-9667 ("Collins II").

Collins I describes the rescue of a viral RNA from which all viral protein-coding sequences were removed (see Abstract; Figure 1 A). The viral RNA described in Collins I encodes a reporter gene, CAT, flanked by the leader and trailer region of RSV. In contrast to the presently claimed viruses, however, the viral RNA described in Collins I does not encode

any viral gene products, including those required to render the virus replication competent. Thus, Collins I can not anticipate the replication competent viruses of the claimed invention.

Collins II describes the construction of a cDNA encoding a truncated version of RSV. The truncated version contained 49.3% of the full length genome of RSV. The construct encoding the truncated version of RSV is shown in Figure 1 D of Collins II. Several open reading frames that are essential for viral replication are missing from the construct, *i.e.*, the N and the P gene. Thus, the viral particles of Collins II encoded by 49.3% of the RS viral genome are not replicating viruses. Collins II does not anticipate the claimed invention since it does not teach all the elements of the claimed invention.

Thus, the rejection of claims 7, 10, and 11 under 35 U.S.C. § 1.102(b) should be withdrawn.

The Rejections under 35 U.S.C. § 102(a) Should Be Withdrawn

Claims 7, 10, and 11 were rejected under 35 U.S.C. § 102(a) as allegedly anticipated by Crowe *et al.*, 1994, Vaccine 12:691-699 ("Crowe"), and claim 11 was rejected under 35 U.S.C. § 102(a) as allegedly anticipated by Conzelmann *et al.*, 1994, J. Virol. 68:713-719 ("Conzelmann") or by Schnell *et al.*, 1994, EMBO J. 13:4195-4203 ("Schnell"). Applicants submit a declaration by Dr. David K. Clarke under 37 CFR § 1.131 (the "Clarke Declaration") to establish a date of invention prior to the publication date of Crowe, Conzelmann and Schnell. Conzelmann has the earliest publication date, January 10, 1994, *i.e.*, the mailing date of the February 1994 issue of the Journal of Virology (see Exhibit 2). In particular, the Clarke Declaration provides evidence to show that Applicants had a date of invention prior to the publication date of Crowe, Conzelmann and Schnell. Thus, the Crowe, Conzelmann and Schnell references are not available as prior art under 35 U.S.C. § 102(a) against claims 7, 10, and 11.

The rejection of claims 7, 10, and 11 under 35 U.S.C. § 1.102(a) should therefore be withdrawn.

No fee is believed to be required for this response. However, should any fee be due, please charge the required amount to Jones Day Deposit Account No. 503013.

Respectfully submitted,

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Virology 283, 59–68 (2001)
doi:10.1006/viro.2001.0894, available online at http://www.idealibrary.com on IDEAL®

Exhibit 1

Chimeric Subgroup A Respiratory Syncytial Virus with the Glycoproteins Substituted by Those of Subgroup B and RSV without the M2-2 Gene Are Attenuated in African Green Monkeys

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Received November 17, 2000; returned to author for revision December 28, 2000; accepted February 23, 2001

Using the existing reverse genetics system developed for the subgroup A respiratory syncytial virus (RSV), a chimeric virus (designated rA-G_BF_B) that expresses subgroup B-specific antigens was constructed by replacing the G and F genes of the A2 strain with those of the 9320 strain of subgroup B RSV. rA-G_BF_B grew well in tissue culture, but it was attenuated in the respiratory tracts of cotton rats and African green monkeys. To further attenuate this chimeric RSV, the M2-2 open reading frame was removed from rA-G_BF_B. rA-$G_BF_B\Delta$M2-2 was highly attenuated in replication in the respiratory tracts of the infected monkeys, but it provided complete protection against wild-type subgroup B RSV challenge following two doses of infection. In this study, rA2ΔM2-2 (a recombinant A2 RSV that lacks the M2-2 gene) was also evaluated in African green monkeys. The replication of rA2ΔM2-2 was highly restricted in both the upper and lower respiratory tracts of the infected monkeys and it induced titers of serum anti-RSV neutralizing antibody that were slightly lower than those induced by wild-type rA2. When rA2ΔM2-2-infected monkeys were challenged with wild-type A2 virus, the replication of the challenge virus was reduced by approximately 100-fold in the upper respiratory tract and 45,000-fold in the lower respiratory tracts. rA2ΔM2-2 and rA-$G_BF_B\Delta$M2-2 could represent a bivalent RSV vaccine composition for protection against multiple strains from the two RSV subgroups.

Key Words: recombinant respiratory syncytial virus; RSV vaccines; chimeric RSV; M2-2 deletion mutants; cotton rats; African green monkeys.

INTRODUCTION

Respiratory syncytial virus (RSV) is the leading cause of serious viral respiratory infection in infants and children worldwide. Despite decades of investigation, no safe and effective vaccines are available to prevent diseases caused by RSV infection. A number of live attenuated RSV candidate vaccines, generated by cold passage and/or chemical mutagenesis, have been evaluated in animals and humans (Crowe *et al.*, 1996a; Friedewald *et al.*, 1968; Gharpure *et al.*, 1969; Hsu *et al.*, 1995; Kim *et al.*, 1971; Richardson *et al.*, 1977). These previous vaccine candidates have been inadequately attenuated and in some circumstances genetically unstable, rendering them unsafe for young children (Hodes *et al.*, 1974; Kim *et al.*, 1973; Wright *et al.*, 1976, 2000). Recently, using the reverse genetics system developed for RSV (Collins *et al.*, 1995; Jin *et al.*, 1998), a large panel of cDNA-derived attenuated RSV have been obtained (reviewed by Collins *et al.*, 1999). A number of these attenuated RSV strains are currently being evaluated for use as vaccines.

RSV is an enveloped virus and contains a single-stranded, negative-sense RNA genome of 15,222 nucleotides (nt). Ten subgenomic mRNAs are encoded by the viral genome and are translated into 11 proteins: the nucleoprotein (N), the phosphoprotein (P), the major polymerase protein (L), the matrix protein (M), the glycoprotein (G), the fusion protein (F), two nonstructural proteins (NS1 and NS2), the small hydrophobic protein (SH), and the M2-1 and M2-2 proteins.

The G and F proteins are the major RSV surface antigens that elicit neutralizing antibodies *in vivo*. Two antigenically diverse RSV subgroups (A and B) have been distinguished on the basis of antigenic and sequence divergence. Within either subgroup, the G and F proteins exhibit high degrees of antigenic similarity. However, between subgroups, extensive differences are observed for the G protein. The antigenic diversity for the G protein between the two subgroups can be as great as 95% (Johnson *et al.*, 1987b). The F glycoprotein is relatively more conserved between the two subgroups. Although there is 91% identity between the amino acid sequences of the subgroup A and B F protein, the antigenic diversity can differ by as much as 50% (Johnson *et al.*, 1987a). This antigenic diversity enables both subgroups to circulate in a community at the same time (Reviewed by McIntosh and Chanock, 1990; Sullender, 2000). Although infection with subgroup A or B RSV in experimental animals induces a high level of resistance against replication of homologous or heterologous sub-

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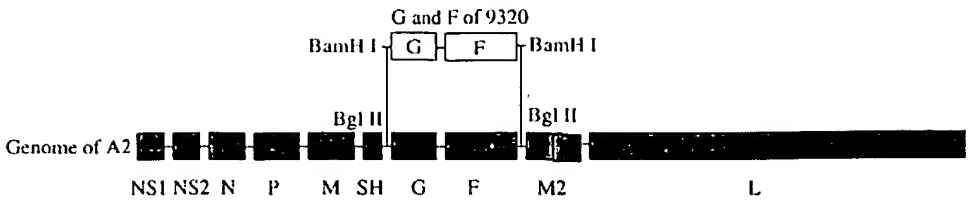

FIG. 1. Insertion of the G and F genes of RSV 9320 strain into recombinant A2 strain. The G and F genes of RSV 9320 were amplified by RT/PCR using primers that contained the introduced *Bam*HI restriction enzyme sites. The DNA cassette containing the G and F genes of 9320 was cloned into the *Bgl*II restriction sites that were created in the SH-G and F-M2 intergenic regions of the A2 strain.

group RSV, infection with attenuated RSV induced better protection against homotypic virus than heterotypic RSV (Crowe *et al.*, 1997a). Recent studies of a RSV vaccine in young infants have shown that infants develop dominant immune response against the RSV G protein than the F protein (Wright *et al.*, 2000). All these available data suggest that prevention of serious RSV diseases through vaccination would require bivalent vaccines containing antigenic components from both subgroups.

Previously, we described a reverse genetics system to generate recombinant RSV from cDNA (Jin *et al.*, 1998) and have used this technology to attenuate subgroup A RSV (Jin *et al.*, 2000a,b). To expedite the development of an attenuated subgroup B RSV vaccine, we used the infectious cDNA developed for the A2 strain to express heterologous subgroup B specific antigens. The construction of a chimeric RSV that expressed an additional G protein from a subgroup B virus in a recombinant A2 virus was described earlier (Jin *et al.*, 1998). Here we describe a recombinant chimeric RSV, designated rA-G_BF_B, in which the G and F genes of subgroup A were replaced with those of subgroup B. This virus is designated rA-G_BF_B. In addition, the M2-2 gene was removed from rA-G_BF_B, designated rA-$G_BF_B\Delta$M2-2, and this virus was shown to be more attenuated than rA-G_BF_B.

African green monkeys (AGM) were evaluated as a nonhuman primate model for assessing the attenuation, immunogenicity, and protective efficacy of RSV vaccine candidates. We showed that rA2 replicated to high titers in both the upper and the lower respiratory tracts of AGM, whereas rA2ΔM2-2, rA-G_BF_B, and rA-$G_BF_B\Delta$M2-2 replicated poorly in the respiratory tracts of monkeys. However, they all induced sufficient immunity to protect animals from experimental challenge.

RESULTS

Construction of cDNA and recovery of RSV A/B chimeric virus

Previously, we constructed an infectious antigenomic cDNA encoding wt RSV strain A2 and its derivative bearing a deletion of the M2-2 gene. Here, these cDNAs were modified by replacing the G and F genes of the A2 strain with those of the subgroup B RSV 9320 strain to produce chimeric viruses expressing RSV subgroup B antigens. The gene-start and gene-end sequences are very conserved between the two RSV subgroups. Therefore, the complete G and F genes of 9320 including their own gene-start and gene-end signals were transferred to the A2 cDNA backbone (Fig. 1). The cDNA encoding the G and F genes of 9320 was obtained by RT/PCR and confirmed by sequence analysis. The constructed chimeric cDNA was designated pRSVA-G_BF_B. pRSVA-$G_BF_B\Delta$M2-2 was constructed by deleting the M2-2 gene from pRSVA-G_BF_B. The M2 gene containing the deletion of the M2-2 open reading frame from rA2ΔM2-2 (Jin *et al.*, 2000a) was introduced into pRSVA-G_BF_B through the unique *Msc*I and *Bam*HI restriction enzyme sites. Both chimeric viruses (rA-G_BF_B and rA-$G_BF_B\Delta$M2-2) were recovered from cDNA using the previously described rescue system (Jin *et al.*, 1998). The recovered recombinant viruses were plaque-purified and amplified in Vero cells.

Characterization of the recombinant chimeric viruses *in vitro*

Expression of the subgroup specific proteins by the chimeric viruses was analyzed by Northern and Western blotting. Using strain-specific probes, 9320-specific G and F mRNAs were detected in cells infected with rA-G_BF_B and rA-$G_BF_B\Delta$M2-2 (Fig. 2A). The M2-2 gene was not detected in cells infected with rA-$G_BF_B\Delta$M2-2 (lane 5), confirming that the M2-2 gene was deleted from this chimeric virus. The 9320 strain-specific protein expression of the two chimeric viruses was also compared with that of rA2, rA2ΔM2-2, and wild-type 9320 (Fig. 2B). The F1 protein of rA-G_BF_B and rA-$G_BF_B\Delta$M2-2 showed the same rate of migration mobility as that of 9320; both migrated faster than that of A2. Western blotting analysis using strain-specific monoclonal antibodies confirmed that the G protein of subgroup B was expressed by rA-G_BF_B and rA-$G_BF_B\Delta$M2-2 (Fig. 2B). Immunoprecipitation using a polyclonal antibody specific to the M2-2 protein further confirmed the ablation of the M2-2 gene in rA2ΔM2-2 and rA-$G_BF_B\Delta$M2-2. The M2-2 protein of RSV strain 9320 was not detected by the polyclonal antiserum raised against the M2-2 protein of strain A2 as there is only 62% homology between the M2-2 proteins of the two RSV subgroups.

Replication of chimeric viruses, rA-G_BF_B and rA-

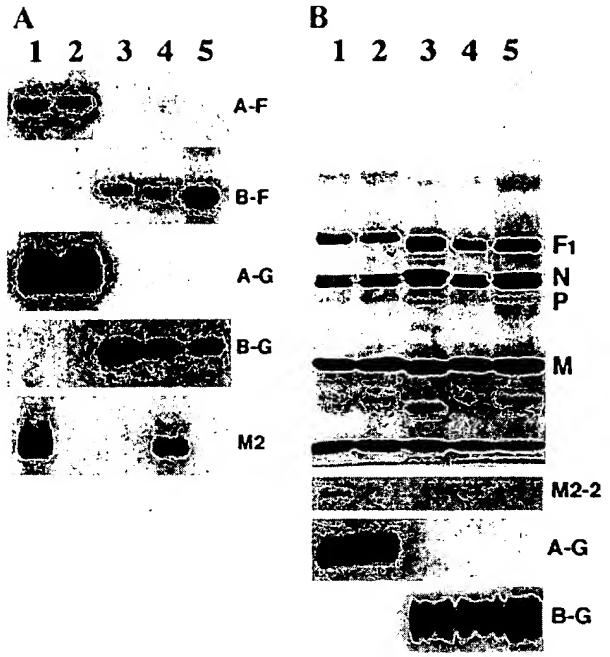

FIG. 2. Strain-specific expression of the chimeric RSV rA-G_BF_B and rA-G_BF_BΔM2-2. (A) Viral RNA expression. Total cellular RNA were extracted from virus-infected Vero cells and the Northern blots were hybridized with probes specific to the G or F gene of either subgroup A or subgroup B RSV. The M2-2 gene expression was examined by using a riboprobe specific to the M2-2 open reading frame. (B) Viral protein expression. The infected Vero cells were labeled with ^{35}S-methionine and ^{35}S-cysteine and the cell lysate immunoprecipitated with anti-RSV polyclonal antibody or anti-M2-2 antibody. To detect the G protein expression, the infected cell extracts were subjected to Western blotting using subgroup-specific monoclonal antibody against the G protein. Both rA-G_BF_B and rA-G_BF_BΔM2-2 expressed the subgroup B-specific G and F proteins and retained normal expression of the other genes derived from the subgroup A2 backbone. No M2-2 protein was expressed in rA2ΔM2-2 and rA-G_BF_BΔM2-2 infected cells. The M2-2 protein of strain 9320 was not recognized by the polyclonal serum raised against the M2-2 protein of strain A2. Lane 1: rA2; lane 2: rA2ΔM2-2; lane 3: 9320; lane 4: rA-G_BF_B; lane 5: rA-G_BF_BΔM2-2.

G_BF_BΔM2-2, was compared to rA2 and rA2ΔM2-2 in both the HEp-2 and the Vero cells (Fig. 3). In Vero cells, infected at an m.o.i. of 0.1, both rA-G_BF_B and rA-G_BF_BΔM2-2 reached peak titers similar to that seen with wild-type rA2 and rA2ΔM2-2, respectively. At a lower m.o.i. of 0.01, the peak titer of rA-G_BF_B was slightly reduced compared to rA2; the level of replication of rA-G_BF_BΔM2-2 was reduced by about 10-fold compared to rA-G_BF_B. In HEp-2 cells, at m.o.i. of 0.1, rA-G_BF_B showed a slightly lower peak titer compared to wt A2, whereas the replication of rA-G_BF_BΔM2-2 was reduced by about 100-fold. At m.o.i. of 0.01, the peak titer of rA-G_BF_B was reduced by about 10-fold compared to rA2 and the peak titer of rA-G_BF_BΔM2-2 was reduced by 100-fold. Therefore, similar to that observed for rA2ΔM2-2, rA-G_BF_BΔM2-2 also exhibited restricted replication in HEp-2 cells, whereas its replication in Vero cells was less impaired.

Replication of chimeric RSV in cotton rats

Cotton rats are susceptible to both subgroup A and subgroup B RSV infection. The levels of replication of rA-G_BF_B and rA-G_BF_BΔM2-2 in the nasal turbinates and lungs of cotton rats were compared with rA2, rA2ΔM2-2, and wild-type 9320 (Table 1). The replication of rA-G_BF_B was below the limit of detection by plaque assay in the nasal turbinates; its replication in lung tissue was reduced by about 3.6 $\log_{10}$ compared to wild-type 9320 and by about 2.0 $\log_{10}$ relative to rA2. The replication of rA2ΔM2-2 was not detected in the nasal turbinates and was 1.6 log lower in the lung compared to rA2. Removal of M2-2 from rA-G_BF_B further attenuated the chimeric virus. No virus replication was detected in either the nasal turbinates or the lungs of cotton rats infected with rA-G_BF_BΔM2-2.

Although rA-G_BF_B and rA-G_BF_BΔM2-2 were attenuated in cotton rats, both chimeric viruses induced sufficient immunity to protect the animals from homologous and heterologous RSV challenge (Table 1). rA-G_BF_BΔM2-2 induced complete protection against subgroup B RSV challenge, but its protection against the heterotypic subgroup A RSV challenge was incomplete in cotton rats. A low level of A2 challenge virus replication was detected in the nasal turbinates of cotton rats previously infected with rA-G_BF_BΔM2-2. The level of serum anti-RSV neutralizing antibody induced by rA-G_BF_B was 2.85-fold lower relative to that induced by wild-type 9320. Serum anti-RSV neutralizing antibody induced by rA-G_BF_BΔM2-2 was approximately fourfold lower compared to that induced by 9320 and 1.5-fold lower than that of rA-G_BF_B. By comparison, the level of serum anti-RSV neutralizing antibody induced by rA2ΔM2-2 was similarly reduced by approximately twofold compared to that of rA2.

Replication of wt RSV and rA2ΔM2-2 in AGM

To investigate RSV attenuation and immunogenicity in primates, replication of recombinant RSV was further studied in AGM. Study A examined the replication of recombinant A2 and wild-type A2 virus in the respiratory tracts of AGM. RSV seronegative AGM were infected with 5.5 $\log_{10}$ pfu of rA2 or wt A2 intranasally and intratracheally and virus shedding was monitored over a period of 12 days in both the upper and the lower respiratory tracts. As shown in Table 2, rA2 replicated well in both the upper and the lower respiratory tracts of AGM. rA2 reached a peak titer of 4.18 and 4.28 $\log_{10}$ pfu/ml at each site, respectively, and shed virus over the same length of time as the wild-type A2 virus (Table 2, study A), though the peak titer of rA2 in the respiratory tracts of AGM was slightly lower than that obtained for wild-type A2 virus. Having confirmed a high level of replication of rA2 in AGM, rA2ΔM2-2 was evaluated for its attenuation, immunogenicity, and protective efficacy in AGM. In a separate study (study B, Table 2), rA2ΔM2-2 showed a

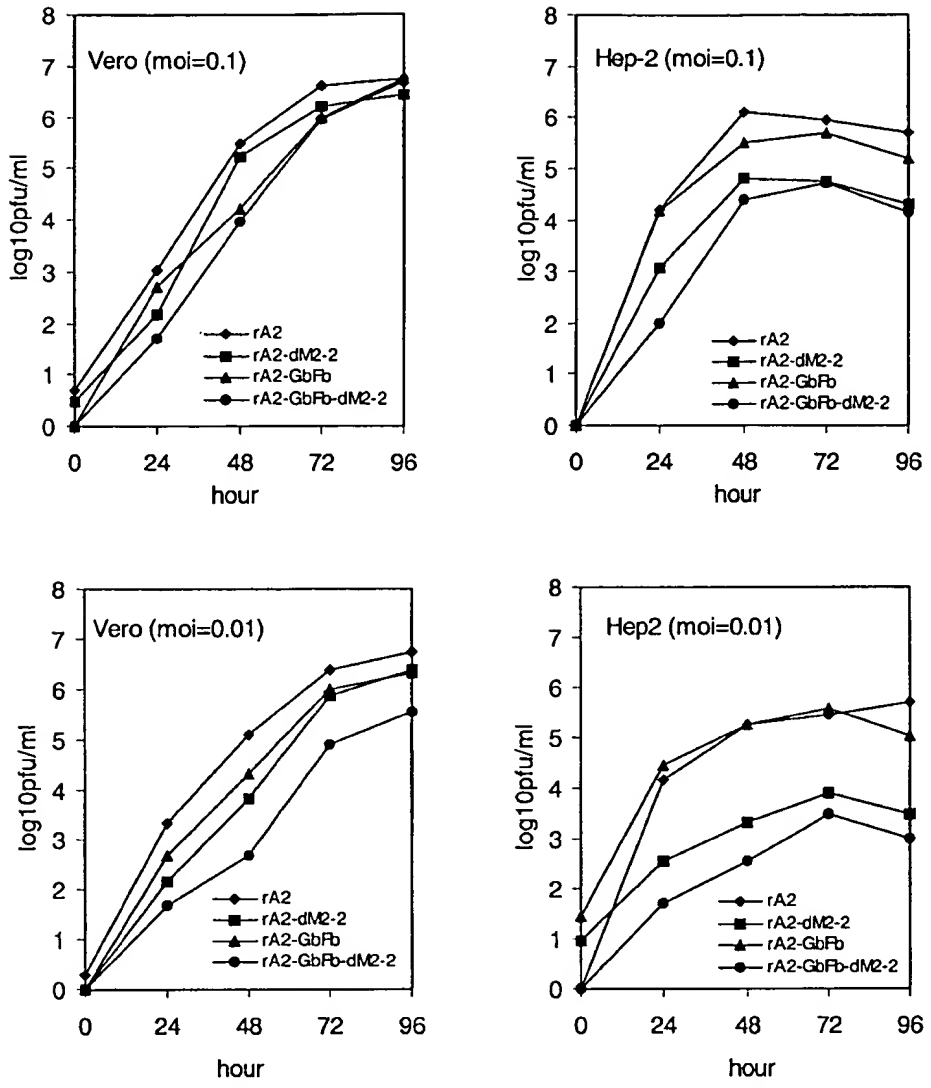

FIG. 3. Growth kinetics of the chimeric viruses in Hep-2 and Vero cells. Hep-2 or Vero cells were infected with viruses in duplicates at an m.o.i. of either 0.1 or 0.01. At 24 h intervals, the infected culture supernatants were harvested and virus titers determined by plaque assay in Vero cells.

greatly reduced level of replication in both the nasopharynx and the trachea compared to rA2. Its peak titer in nasopharynx was reduced by 3.1 $\log_{10}$, whereas the peak titer in the trachea was reduced by 3.25 $\log_{10}$ compared to rA2. Despite the much lower level of replication in the respiratory tracts, rA2ΔM2-2 induced a significant level of serum anti-RSV neutralizing antibody. The antibody titer induced by rA2ΔM2-2 was about fourfold lower than that induced by rA2 at 3 weeks postinfection (Table 3). When challenged with wild-type A2 virus, rA2ΔM2-2 provided partial protection against wild-type RSV replication in the upper respiratory tract and much greater protection in the lower respiratory tract of immunized monkeys. Monkeys previously infected with rA2 were fully protected against wt A2 virus replication in both the upper and the lower respiratory tracts (Table 3). Although rA2ΔM2-2 did not provide complete protection in the respiratory tracts of immunized monkeys, it reduced virus shedding by 5 days. Two weeks after challenge, the level of serum anti-RSV neutralizing antibody from rA2ΔM2-2 infected monkeys approached that induced by rA2.

Replication of chimeric RSV and wild-type 9320 in AGM

We next compared the level of replication of chimeric rA-G_BF_B with that of wild-type 9320. RSV seronegative AGM were inoculated with 5.5 $\log_{10}$ pfu of rA-G_BF_B or 9320 by intranasal and intratracheal instillation. The throat swab and tracheal lavage samples were collected over 12 days for virus quantitation. 9320 replicated to a level similar to that of wild-type A2 virus (Table 2). The peak titer of rA-G_BF_B at both sites of the respiratory tracts of the infected monkeys was about 1000-fold reduced compared to that of 9320. Animals infected with rA-G_BF_B shed virus for a shorter period of time than those infected with 9320. Despite its significantly attenuated replication, rA-G_BF_B provided complete protection when challenged with wild-type 9320. No challenge virus was detected in

TABLE 1

Replication, Immunogenicity, and Protection of Recombinant RSV against wt RSV Infection in the Upper and Lower Respiratory Tracts of Cotton Rats

Virus[a]	Virus titer[b] (mean $\log_{10}$pfu/g ± SE)		Neutralizing Ab titer (mean reciprocal $\log_2$)[c]	Titer of challenge virus (mean $\log_{10}$pfu/g ± SE)[d]			
				A2		9320	
	NT	Lung		NT	Lung	NT	Lung
rA2	3.9 ± 0.13	3.57 ± 0.07	10.0	<1.4	<1.4	ND[e]	ND
rA2ΔM2-2	<1.4	2.02 ± 0.12	9.0	<1.4	<1.4	ND	ND
Control	<1.4	<1.4	<3.3	4.2 ± 0.14	6.0 ± 0.06	2.3 ± 0.53	5.2 ± 0.01
9320	2.8 ± 0.57	5.6 ± 0.05	10.64	<1.4	<1.4	<1.4	<1.4
rA-G_BF_B	<1.4	1.94 ± 0.31	9.13	<1.4	<1.4	<1.4	<1.4
rA-G_BF_BΔM2-2	<1.4	<1.4	8.57	1.2 ± 0.65	<1.4	<1.4	<1.4

[a] Cotton rats were administered with 5.5 $\log_{10}$PFU of virus intranasally under light anesthesia on day 0 and sacrificed on day 4.

[b] Virus titers from the nasal turbinates (NT) and lung tissues were determined by plaque assay.

[c] Serum RSV neutralizing antibody titers were determined by a complement-enhanced 50% plaque reduction assay with wt A2 or 9320.

[d] On day 21 of virus infection, cotton rats in groups of six were challenged with wt A2 or wt 9320 and the challenge virus titers from the nasal turbinates (NT) and lung tissues were determined by plaque assay.

[e] ND, not determined.

either the upper or the lower respiratory tracts of the monkeys previously immunized with rA-G_BF_B (Table 3). Consistent with the level of protection seen in monkeys immunized with rA-G_BF_B, the level of serum anti-RSV neutralizing antibody from these monkeys was similar to that observed for wild-type 9320-infected animals.

rA-G_BF_BΔM2-2 was evaluated in AGM in a separate study (study C). The replication of rA-G_BF_BΔM2-2 was not detected in the upper respiratory tracts and a very low level of virus replication was detected in the lower respiratory tracts of the infected monkeys (Table 2). Since rA-G_BF_BΔM2-2 appeared to be more attenuated than rA-G_BF_B and rA2-ΔM2-2, an additional boosting dose was administered 4 weeks later. The boosting infection greatly augmented immune response and provided complete protection against wild-type 9320 RSV challenge. The level of serum anti-RSV neutralizing antibody induced by rA-G_BF_BΔM2-2 was about fourfold lower than that induced by rA-G_BF_B. However, after a second dose of boosting infection, the level of serum neutralizing antibody was increased by about eightfold and it was further augmented by an additional twofold following subsequent wild-type RSV infections.

DISCUSSION

In an attempt to develop live attenuated RSV vaccine, we are using a recently developed reverse genetics system to attenuate RSV by introducing various mutations into the RSV genome. This approach has generated a number of attenuated subgroup A recombinant RSV by different groups (Jin *et al.*, 2000a,b; Teng and Collins, 1999; Teng *et al.*, 2000; Bermingham and Collins, 1999; Whitehead *et al.*, 1999a,b). To expedite vaccine develop-

TABLE 2

Replication of Recombinant RSV in the Upper and Lower Respiratory Tracts of African Green Monkeys

Virus[a]	AGM number	Virus shedding (days)	Virus peak titer (Mean $\log_{10}$pfu ± SE)[b]	
			Nasopharyngeal swab	Tracheal lavage
wt A2	4 (Study A)	8	4.67 ± 0.17	4.97 ± 0.04
rA2	4 (Study A)	8	4.18 ± 0.18	4.28 ± 0.27
rA2	4 (Study B)	9	3.44 ± 0.27	3.91 ± 0.18
rA2ΔM2-2	4 (Study B)	4	0.33 ± 0.26	0.66 ± 0.40
9320	4 (Study B)	9	4.51 ± 0.18	4.36 ± 0.45
rA-G_BF_B	4 (Study B)	4	1.50 ± 0.42	1.77 ± 0.25
rA-G_BF_BΔM2-2	4 (Study C)	3	<0.7	0.25 ± 0.25

[a] African green monkeys were administered with 5.5 $\log_{10}$PFU of virus intranasally and intratracheally. Nasopharyngeal swab samples were collected daily for 12 days, and tracheal-lavage samples were collected on days 3, 5, 7, and 10.

[b] Virus titers were determined in the nasopharyngeal swab and tracheal-lavage by plaque assay and only the peak titers are shown.

TABLE 3

Evaluation of Recombinant RSV for Their Levels of Immunogenicity and Efficacy against Wild Type Challenge Virus in African Green Monkeys

Virus	Challenge virus[a]	Virus peak titer (Mean $\log_{10}$pfu ± SE)[b]		Neutralizing Ab titer (Mean reciprocal $\log_2$)[c]		
		Nasopharyngeal swab	Tracheal lavage	Day 0	Day 28	Day 42
rA2	A2	<0.7	<0.7	<3.3	9.7	10.5
rA2ΔM2-2	A2	2.64 ± 0.07	0.46 ± 0.47	<3.3	7.7	9.25
None	A2	4.67 ± 0.17	4.97 ± 0.04	<3.3	<3.3	10.5
9320	9320	<0.7	<0.7	<3.3	7.0	10.0
rA-G_BF_B	9320	<0.7	<0.7	<3.3	7.75	10.5
rA-G_BF_BΔM2-2[d]	9320	<0.7	<0.7	<3.3	5.5	8.75[d]
None	9320	4.51 ± 0.18	4.36 ± 0.35	<3.3	<3.3	10.0

[a] African green monkeys were administered with 5.5 $\log_{10}$PFU of virus intranasally and intratracheally and on day 28; monkeys were challenged with wt A2 or wt 9320 at a dose of 5.5 $\log_{10}$PFU intranasally and intratracheally.

[b] Nasopharyngeal swab samples were collected daily for 10 days, and tracheal-lavage samples were collected on days 3, 5, 7, and 10. Challenge virus titers were determined by plaque assay. Only the peak titers are shown.

[c] Serum RSV neutralizing antibody titers from monkeys infected with rA2 and rA2ΔM2-2 before challenge infection (day 28) and 14 days post challenge (day 42) were determined by a complement-enhanced 50% plaque reduction assay with wt A2. The neutralizing antibody titers from monkeys infected with 9320, rA-G_BF_B, and rA-G_BF_BΔM2-2 were determined by microneutralization assay (Cheng *et al.*, manuscript in preparation).

[d] An additional boosting dose was administered; shown here is postboosting neutralizing antibody titer at day 56. The antibody titer after challenge (day 70) was 9.75 $\log_2$.

ment for subgroup B RSV, we used recombinant A2 virus as a vector to express subgroup B RSV surface antigens. The chimeric virus should elicit a balanced immune response and provide protection against subgroup B RSV infection. Previously, we described a chimeric virus, A2(B-G), that expressed two G proteins, one derived from A2 strain and the other derived from 9320 strain (Jin *et al.*, 1998). A2(B-G) provided complete protection against subsequent wild-type RSV A and B strains infection in cotton rats. While this chimeric virus retained both G genes of two subgroups during passage in tissue culture, frameshift mutations have been detected at oligo(A) tracts of one or the other subgroup G gene on separate occasions during *in vitro* passages. As an alternative approach to expressing RSV subgroup B antigens, we constructed a different chimeric virus in which the G and F genes of the A2 strain were completely replaced by the G and F genes of the 9320 strain. The chimeric RSV was then further attenuated using a strategy developed for attenuating the A2 virus.

The recovered chimeric RSV (rA-G_BF_B) replicated efficiently in Vero cells, but its growth in HEp-2 cells was reduced by 5- to 10-fold relative to rA2. rA-G_BF_B was attenuated in both the upper and the lower respiratory tracts of cotton rats. To determine whether the attenuation of rA-G_BF_B was host specific, this chimeric virus was further evaluated in AGM that are genetically more closely related to humans than rodents. RSV infection in AGM is less well characterized and there is a wide range in the reported peak titer (Crowe *et al.*, 1996b; Kakuk *et al.*, 1993). Therefore, we first tested RSV infection in AGM using wild-type viruses. We showed that both subgroup A and subgroup B RSV replicated equally well in AGM and virus titers recovered from the upper and lower respiratory tracts of AGM approached those observed in infected chimpanzees (Crowe *et al.*, 1994). When rA-G_BF_B was evaluated in AGM, it showed a mean peak titer reduction of 3.0 $\log_{10}$ in the upper respiratory tract and a reduction of 2.59 $\log_{10}$ in the lower respiratory tract.

The level of attenuation of rA-G_BF_B in AGM was consistent with what we observed in cotton rats. However, this result was somewhat different from that reported for a recently described chimeric RSV in which the G and F genes of A2 were replaced with those of RSV B1 strain (rAB1) (Whitehead *et al.*, 1999b). Though rAB1 and rA-G_BF_B are similarly attenuated in cotton rats, rAB1 was not attenuated in chimpanzees. In contrast to rA-G_BF_B, rAB1 replicated better than wt RSV B1 in both the upper and the lower respiratory tracts of chimpanzees (Whitehead *et al.*, 1999b). Part of this discrepancy may be explained by the semipermissiveness of chimpanzees to wild-type subgroup B RSV infection. However, there exists the possibility that rA-G_BF_B is more attenuated than rAB1 because of differences in the subgroup B strain surface antigens or constellation effects when these antigens are introduced into an A2 background. Chimerization of surface antigens resulting in an attenuated virus has been reported for several paramyxoviruses. A chimeric measles virus with the HN and F proteins replaced by the G protein of VSV was highly restricted in replication *in vitro* (Spielhofer *et al.*, 1998). A chimeric Rinderpest virus in which the F and H proteins were replaced by the heterologous surface proteins of a closely related pestedes-petits-ruminants virus was attenuated *in vitro*, as indicated by slow virus growth and low virus yield (Das *et al.*, 2000). Most recently, it was reported that the PIV3-

PIV2 chimeric virus, in which the F and HN genes of PIV3 were replaced by those of PIV2, was not attenuated *in vitro*, but it was highly attenuated in hamsters, AGM, and chimpanzees (Tao *et al.*, 2000). On the other hand, the chimeric PIV3-PIV1 was not attenuated *in vivo* (Tao *et al.*, 1998, 1999). Thus, it appears that chimerization of different heterologous proteins can result in different phenotypes. Though attenuated in AGM, rA-G_BF_B induced significant levels of anti-RSV neutralizing antibody and provided complete protection against subsequent challenge with wild-type subgroup B RSV.

We previously reported that the recombinant A2 RSV lacking the M2-2 gene is attenuated in mice and cotton rats. In this study, we evaluated rA2ΔM2-2 for its attenuation, immunogenicity, and protection against wild-type RSV challenge in AGM. We showed that rA2ΔM2-2 was attenuated in the respiratory tracts of AGM and following challenge, much reduced replication of wild-type RSV was observed in animals previously infected with rA2ΔM2-2. The protection was higher in the lower respiratory tract than the upper respiratory tract. The level of replication and protection observed for rA2ΔM2-2 in AGM is very similar to that reported in a chimpanzee study for a similar recombinant RSV that had the M2-2 protein expression silenced (Bermingham and Collins, 1999; Teng *et al.*, 2000). rA2ΔM2-2 may prove to be more attenuated in humans than a previously tested vaccine candidate cpts248/404 (Teng *et al.*, 2000). cpts248/404 was neither sufficiently attenuated nor genetically stable in naive infants (Crowe *et al.*, 1994; Wright *et al.*, 2000). The serum anti-RSV neutralizing antibody titer induced by rA2ΔM2-2 was slightly lower than that induced by the wild-type RSV infection. However, the augmentation of neutralizing antibody titer after the challenge suggests that the immunogenicity of rA2ΔM2-2 could be enhanced by repeat administrations.

Since rA2ΔM2-2 exhibits many of the desired features in a live attenuated vaccine, we considered the deletion of the M2-2 gene an appropriate way to further attenuate the chimeric rA-G_BF_B. *In vitro* studies indicated that rA-G_BF_BΔM2-2 had phenotypes similar to rA2ΔM2-2, exhibiting increased syncytial formation, reduced growth in HEp-2 cells, and unbalanced RNA transcription to replication. rA-G_BF_BΔM2-2 was more attenuated than rA-G_BF_B and rA2ΔM2-2 in both cotton rats and AGM. This attenuated replication in the host led to its reduced immunogenicity. Thus, although rA-G_BF_B provided complete protection to both subgroup A and subgroup B RSV challenge, A-G_BF_BΔM2-2 provided lower protection to subgroup A virus infection than to subgroup B strain infection in cotton rats. A very low level of serum anti-RSV neutralizing antibody was detected in monkeys infected with rA-G_BF_BΔM2-2. The second dose administration of A-G_BF_BΔM2-2 greatly augmented antibody response and provided complete protection against subsequent experimental challenge in AGM. Our data implied that a live attenuated RSV vaccine containing components from both subgroups need to be administered in multiple doses to achieve a higher level of durable immunity. rA-G_BF_BΔM2-2, in combination with rA2ΔM2-2, may represent suitable vaccines for protecting against both subgroup A and subgroup B RSV infections.

MATERIALS AND METHODS

Cells and viruses

Monolayer cultures of HEp-2 and Vero cells (obtained from American Type Culture Collections, ATCC) were maintained in minimal essential medium (MEM) containing 5% fetal bovine serum (FBS). Wild-type RSV strains, A2 and 9320, were obtained from ATCC and grown in Vero cells. Modified vaccinia virus Ankara (MVA-T7) expressing bacteriophage T7 RNA polymerase was provided by Dr. Bernard Moss and grown in CEK cells.

Construction of chimeric cDNA clone

The wild-type RSV strain 9320, originally isolated in Massachusetts in 1977 and classified as subgroup B RSV (Hierholzer and Hirsch, 1979), was used in this study. The 9320 RSV was grown in Vero cells and the viral RNA was extracted from infected cell culture supernatant. A cDNA fragment containing the G and F genes of RSV 9320 was obtained by RT/PCR using the following primers: ATCA*GGATCC*<u>*ACAATAACATTGGGGCAAATGCAACC*</u> and CTGGCATTC*GGATCC*<u>*GTTTTATGTAACTATGAGTTG*</u> (the *Bam*HI sites engineered for cloning are in italics and 9320 specific sequences are underlined). *Bam*HI restriction enzyme sites were introduced upstream of the gene start sequence of G and downstream of the gene end sequence of F. The PCR product was first introduced into the T/A cloning vector (Invitrogen) and the sequences were confirmed by DNA sequencing. The *Bam*HI restriction fragment containing the G and F gene cassette of 9320 was then transferred into a RSV cDNA subclone pRSV(R/H) that contained RSV sequences from nt 4326 to nt 9721 through the introduced *Bgl*II sites at nt 4655 (upstream of the gene start signal of G) and at nt 7552 (downstream of the gene end signal of F). Introduction of these two *Bgl*II sites were made by PCR mutagenesis using the QuickChange mutagenesis kit (Strategene, La Jolla, CA). *Bam*HI and *Bgl*II restriction enzyme sites have compatible ends but ligation obliterates both restriction sites. The *Xho*I (nt 4477) to *Bam*HI (nt 8498) restriction fragment containing the G and F genes of 9320 was examined by sequencing analysis and then shuttled into the infectious RSV antigenomic cDNA clone pRSVC4G (Jin *et al.*, 1998). The chimeric antigenomic cDNA was designated pRSV-G_BF_B. To delete the M2-2 gene from pRSV-G_BF_B, the Msc I (nt 7692) to *Bam*HI (nt 8498) fragment from rA2ΔM2-2 which contained the

M2-2 deletion (Jin *et al.*, 2000a) was introduced into pRSV-G_BF_B. The chimeric cDNA clone that lacks the M2-2 gene was designated pRSV-$G_BF_B\Delta$M2-2.

Recovery of recombinant RSV

Recovery of recombinant RSV from cDNA was described previously (Jin *et al.*, 1998). Briefly, HEp-2 cells in six-well plates at 80% confluence were infected with MVA at an m.o.i. of 5 pfu/cell for 1 h and then were transfected with full-length antigenomic plasmids (pRSV-G_BF_B or pRSV-$G_BF_B\Delta$M2-2), together with plasmids expressing the RSV N, P, and L proteins using LipofecTACE reagent (Life Technologies, Gaithersburg, MD). After incubating the transfected cells at 35°C for 3 days, the culture supernatants were passaged in Vero cells for 6 days to amplify rescued virus. The recovered recombinant viruses were biologically cloned by three successive plaque purifications and further amplified in Vero cells. Virus recovered from pRSV-G_BF_B-transfected cells was designated rA-G_BF_B and that from pRSV-$G_BF_B\Delta$M2-2 transfected cells was designated rA-$G_BF_B\Delta$M2-2. Virus titer was determined by plaque assay and plaques were enumerated by immunostaining using polyclonal anti-RSV A2 serum (Biogenesis, Sandown, NH).

Virus characterization

The expression of viral RNA for each recovered chimeric RSV was analyzed by Northern blotting. Total cellular RNA was extracted from virus-infected cells at 48 h postinfection. The RNA blot was hybridized with a γ-[^{32}P]ATP-labeled oligonucleotide probe specific for the F gene of 9320 (GAGGTGAGGTACAATGCATTAATAG-CAAGATGGAGGAAGA) or a γ-[^{32}P]ATP-labeled probe specific for the F gene of A2 (CAGAAGCAAAACAAAAT-GTGACTGCAGTGAGGATTGTGGT). To detect the G mRNA of the chimeric viruses, RNA blots were hybridized with a 190-nt riboprobe specific to the G gene of 9320 or a 130-nt riboprobe specific to the G gene of A2. Both riboprobes were labeled with α-[^{32}P]-CTP. Hybridization was performed at 65°C in Express Hyb solution (Clontech, Palo Alto, CA) overnight. Membranes were washed at 65°C under stringent conditions and exposed to film.

Viral specific proteins from infected cells were analyzed by immunoprecipitation of the infected cell extracts or by Western blotting. To immunoprecipitate viral proteins, Vero cells were infected with virus at an m.o.i. of 1.0 and labeled with ^{35}S-promix (100 μCi/ml ^{35}S-Cys and ^{35}S-Met; Amersham, Arlington Heights, IL) from 14 to 18 h postinfection. The labeled cell monolayers were lysed with RIPA buffer and the polypeptides immunoprecipitated by polyclonal goat anti-RSV A2 serum (Biogenesis) or by a polyclonal antibody against the M2-2 protein (Jin *et al.*, 2000a). Immunoprecipitated polypeptides were electrophoresed on SDS-PAGE and detected by autoradiography. For Western blotting analysis, virus-infected Vero cells were lysed in protein lysis buffer and the proteins were separated on 12% SDS-PAGE. The proteins were transferred to a nylon membrane and immunoblotting was performed as described (Jin *et al.*, 1997), using a monoclonal antibody recognizing the G protein of strain 9320 or a monoclonal antibody against the G protein of A2 (Storch and Park, 1987).

Growth of chimeric RSV *in vitro* was compared with wild-type recombinant A2 (rA2) and rA2ΔM2-2. Growth-cycle analysis was performed in both HEp-2 and Vero cells. Cells grown in 6-cm dishes were infected with each virus at a m.o.i. of 0.01 or 0.1. After 1 h absorption at room temperature, the infected cell monolayers were washed 3 times with PBS and incubated at 35°C with 4 ml of Opti-MEM in an incubator containing 5% CO_2. At various times postinfection, 200 μl of the culture supernatant was collected and stored at −70°C for virus titration. Each aliquot removed was replaced with an equal amount of fresh medium. Virus titer was determined by plaque assay in Vero cells on 12-well plates using an overlay of 1% methylcellulose and 1× L15 medium containing 2% FBS.

Virus replication in cotton rats

Virus replication *in vivo* was determined in respiratory pathogen-free *Sigmodon Hispidus* cotton rats. Cotton rats in groups of 12 or 18 were inoculated intranasally under light methoxyflurane anesthesia with $10^{5.5}$ pfu of virus per animal in a 0.1-ml inoculum. On day 4 postinoculation, six animals were sacrificed by CO_2 asphyxiation and their nasal turbinates and lungs were harvested separately. Tissues were homogenized and virus titers determined by plaque assay in Vero cells. Three weeks later, the remaining six animals were anesthetized, their serum samples were collected, and a challenge inoculation of 10^6 pfu of biologically derived wild-type RSV strain A2 or 9320 administered intranasally. To investigate the cross-protection of the chimeric viruses to heterologous RSV, six additional animals infected with rA-G_BF_B or rA-$G_BF_B\Delta$M2-2 were also challenged with wt A2 RSV. Four days postchallenge, the animals were sacrificed and both nasal turbinates and lungs were harvested, homogenized, and virus titer determined by plaque assay. Serum neutralizing antibodies against RSV A2 or 9320 strain were determined by a 50% plaque reduction assay (Coates *et al.*, 1966).

Virus replication in AGM

Recombinant RSV was evaluated for their replication, immunogenicity, and protective efficacy in AGM (*Cercopithecus aethiops*). AGM, obtained from St. Kitts with an average age of 4.2 years and body weight ranging from 2.2 to 4.3 kg, were used in the first study (study A) to compare the replication of rA2 with wild-type A2. The

second study (study B) used AGM with ages ranging from 5.3 to 8.4 years and an average body weight of 4.15 kg. None of the monkeys had detectable serum neutralizing antibodies for RSV 9320 or A2 (titer < 1:10). Groups of four monkeys were inoculated with either wild-type A2, rA2, rA2ΔM2-2, wild-type 9320, or rA-G_BF_B by both intranasal and intratracheal route with a dose of $10^{5.5}$ pfu in a 1.0 ml inoculum at each site. Following inoculation, daily nasopharyngeal (NP) swabs were collected from each monkey for 12 days under Telazol anesthesia and bronchoalveolar lavage (BAL) were collected on days 3, 5, 7, and 10 postinfection (Kakuk *et al.*, 1993). On day 28 postinfection, serum samples were collected from each infected monkey and the monkeys were challenged with either wild-type A2 or 9320 at both the intranasal and the intratracheal sites with a dose of $10^{5.5}$ pfu in a 1.0-ml inoculum. Replication of the challenge virus in the upper and lower respiratory tracts of monkeys was examined by quantitation of virus shed in NP and tracheal lavage specimens. The NP samples were collected daily for 10 days and BAL samples were collected on days 3, 5, 7, and 10 postchallenge. Fourteen days after wild-type virus challenge, serum samples were collected for measurement of RSV neutralizing antibody. rA-G_BF_BΔM2-2 was evaluated in a separate study (study C). Four week after infection, a group of four monkeys were administered with an additional boosting dose of 5.5 $\log_{10}$ pfu of rA-G_BF_BΔM2-2 intranasally and intratracheally and monkeys were challenged with 5.5 $\log_{10}$ pfu of wt 9320 virus 4 weeks after boosting infection. NP and BAL samples were collected from rA-G_BF_BΔM2-2-infected monkeys the same as described for those infected with rA-G_BF_B. Serum samples were collected at day 0 (preserum), day 28 (preboosting), day 56 (postboosting), and day 70 (postchallenge). The levels of neutralizing antibody from monkeys infected with rA2 and rA2ΔM2-2 were determined by the 50% plaque reduction assay using wild-type A2 virus. The levels of neutralizing antibody from monkeys infected with 9320, rA-G_BF_B, and rA-G_BF_BΔM2-2 were determined by a microneutralization assay (Cheng *et al.*, manuscript in preparation). The virus shedding in the NP and BAL samples were quantitated by plaque assay using Vero cells.

ACKNOWLEDGMENTS

We thank Adam Seddiqui and Wanda Williams for assistance with the cotton rat studies; the tissue culture facility at Aviron for supplying cells; Drs. Ken Soike, Vicki Ttraina-Dorge, and Richard Rockar of the Tulane regional primate center for performing experiments in AGM; Nick Nguyen for technical assistance; Dr. Kathy Coelingh for advice on primate studies; and Drs. George Kemble, Ann Arvin, and Harry Greenberg for advice and critical review of the manuscript. This work was supported in part by NIH SBIR Grants (2R44AI45267-01/02).

REFERENCES

Bermingham, A., and Collins, P. L. (1999). The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. *Proc. Natl. Acad. Sci. USA* 96(20), 11259–11264.

Coates, H. V., Alling, D. W., and Chanock, R. M. (1966). An antigenic analysis of respiratory syncytial virus isolates by a plaque reduction neutralization test. *Am. J. Epidemiol.* 83(2), 299–313.

Collins, P. L., Hill, M. G., Camargo, E., Grosfeld, H., Chanock, R. M., and Murphy, B. R. (1995). Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. *Proc. Natl. Acad. Sci. USA* 92, 11563–11567.

Collins, P. L., Whitehead, S. S., Bukreyev, A., Fearns, R., Teng, M. N., Juhasz, K., Chanock, R. M., and Murphy, B. R. (1999). Rational design of live-attenuated recombinant vaccine virus for human respiratory syncytial virus by reverse genetics. *Adv. Virus Res.* 54, 423–451.

Crowe, J., Jr., Firestone, C. Y., Whitehead, S. S., Collins, P. L., and Murphy, B. R. (1996a). Acquisition of the ts phenotype by a chemically mutagenized cold-passaged human respiratory syncytial virus vaccine candidate results from the acquisition of a single mutation in the polymerase (L) gene. *Virus Genes* 13(3), 269–273.

Crowe, J. E., Jr., Bui, P. T., Davis, A. R., Chanock, R. M., and Murphy, B. R. (1994). A further attenuated derivative of a cold-passaged temperature-sensitive mutant of human respiratory syncytial viurs (RSV cpts-248) retains immunogenicity and protective efficacy against wild-type challenge in seronegative chimapanzees. *Vaccine* 12, 783–790.

Crowe, J. E., Jr., Bui, P. T., Firestone, C. Y., Connors, M., Elkins, W. R., Chanock, R. M., and Murphy, B. R. (1996b). Live subgroup B respiratory syncytial virus vaccines that are attenuated, genetically stable, and immunogenic in rodents and nonhuman primates. *J. Infect. Dis.* 173, 829–839.

Das, S. C., Baron, M. D., and Barrett, T. (2000). Recovery and characterization of a chimeric rinderpest virus with the glycoproteins of peste-des-petits-ruminants virus: Homologous F and H proteins are required for virus viability. *J. Virol.* 74(19), 9039–9047.

Friedewald, W. T., Forsyth, B. R., Smith, C. B., Gharpure, M. A., and Chanock, R. M. (1968). Low-temperature-grown RS virus in adult volunteers. *J. Am. Med. Assoc.* 203(8), 690–694.

Gharpure, M. A., Wright, P. F., and Chanock, R. M. (1969). Temperature-sensitive mutants of respiratory syncytial virus. *J. Virol.* 3(4), 414–421.

Hierholzer J. C., and Hirsch, M. S. (1979). Croup and pneumonia in human infants associated with a new strain of respiratory syncytial virus. *J. Infect. Dis.* 140, 826–828.

Hodes, D. S., Kim, H. W., Parrott, R. H., Camargo, E., and Chanock, R. M. (1974). Genetic alteration in a temperature-sensitive mutant of respiratory syncytial virus after replication in vivo. *Proc. Soc. Exp. Biol. Med.* 145(4), 1158–1164.

Hsu, K. L., Crowe, J. E., Jr., Lubeck, M. D., Davis, A. R., Hung, P. P., Chanock, R. M., and Murphy, B. R. (1995). Isolation and characterization of a highly attenuated repiratory syncytial virus (RSV) vaccine candidate by mutagenesis of the incompletely attenuated RSV A2 ts-1 NG-1 mutant virus. *Vaccine* 13, 509–515.

Jin, H., Cheng, X., Zhou, H. Z., Li, S., and Seddiqui, A. (2000a). Respiratory syncytial virus that lacks open reading frame 2 of the M2 gene (M2–2) has altered growth characteristics and is attenuated in rodents. *J. Virol.* 74(1), 74–82.

Jin, H., Clarke, D., Zhou, Z.-Y. H., Cheng, X., Coelingh, K., Bryant, M., and Li, S. (1998). Recombinant human respiratory syncytial virus (RSV) from cDNA and construction of subgroup A and B chimeric RSV. *Virology* 251, 206–214.

Jin, H., Leser, G. P., Zhang, J., and Lamb, R. A. (1997). Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape. *EMBO J.* 16(6), 1236–1247.

Jin, H., Zhou, H., Cheng, X., Tang, R., Munoz, M., and Nguyen, N. (2000b). Recombinant respiratory syncytial viruses with deletions in the NS1, NS2, SH, and M2–2 genes are attenuated in vitro and in vivo. *Virology* 273(1), 210–218.

Johnson, P., Jr., Olmsted, R. A., Prince, G. A., Murphy, B. R., Alling, D. W.,

Walsh, E. E., and Collins, P. L. (1987a). Antigenic relatedness between glycoproteins of human respiratory syncytial virus subgroups A and B: Evaluation of the contributions of F and G glycoproteins to immunity. *J. Virol.* 61(10), 3163–3166.

Johnson, P. R., Spriggs, M. K., Olmsted, R. A., and Collins, P. L. (1987b). The G glycoprotein of human respiratory syncytial viruses of subgroups A and B: Extensive sequence divergence between antigenically related proteins. *Proc. Natl. Acad. Sci. USA* 84(16), 5625–5629.

Kakuk, T. J., Soike, K., Brideau, R. J., Zaya, R. M., Cole, S. L., Zhang, J. Y., Roberts, E. D., Wells, P. A., and Wathen, M. W. (1993). A human respiratory syncytial virus (RSV) primate model of enhanced pulmonary pathology induced with a formalin-inactivated RSV vaccine but not a recombinant FG subunit vaccine. *J. Infect. Dis.* 167(3), 553–561.

Kim, H. W., Arrobio, J. O., Brandt, C. D., Wright, P., Hodes, D., Chanock, R. M., and H., P. R. (1973). Safety and antigenicity of temperature-sensitive (ts) mutant respiratory syncytial virus (RSV) in infants and children. *Pediatrics* 52, 56–63.

Kim, H. W., Arrobio, J. O., Pyles, G., Brandt, C. D., Camargo, E., Chanock, R. M., and Parrott, R. H. (1971). Clinical and immunological response of infants and children to administration of low-temperature adapted respiratory syncytial virus. *Pediatrics* 48(5), 745–755.

McIntosh, K., and Chanock, R. M. (1990). Respiratory syncytial virus. In "Virology" 2nd ed. (D. M. Knipe *et al.*, Eds.), Raven Press, New York.

Richardson, L. S., Schnitzer, T. J., Belshe, R. B., Camargo, E., Prevar, D. A., and Chanock, R. M. (1977). Isolation and characterization of further defective clone of a temperature-sensitive mutant (ts-1) of respiratory syncytial virus. *Arch. Virol.* 54, 53–60.

Spielhofer, P., Bachi, T., Fehr, T., Christiansen, G., Cattaneo, R., Kaelin, K., Billeter, M. A., and Naim, H. Y. (1998). Chimeric measles viruses with a foreign envelope. *J. Virol.* 72(3), 2150–2159.

Storch, G. A., and Park, C. S. (1987). Monoclonal antibodies demonstrate heterogeneity in the G glycoprotein of prototype strains and clinical isolates of respiratory syncytial virus. *J. Med. Virol.* 22, 345–356.

Sullender, W. M. (2000). Respiratory syncytial virus genetic and antigenic diversity. *Clin. Microbiol. Rev.* 13(1), 1–15.

Tao, T., Durbin, A. P., Whitehead, S. S., Davoodi, F., Collins, P. L., and Murphy, B. R. (1998). Recovery of a fully viable chimeric human parainfluenza virus (PIV) type 3 in which the hemagglutinin-neuraminidase and fusion glycoproteins have been replaced by those of PIV type 1. *J. Virol.* 72(4), 2955–2961.

Tao, T., Skiadopoulos, M. H., Davoodi, F., Riggs, J. M., Collins, P. L., and Murphy, B. R. (2000). Replacement of the ectodomains of the hemagglutinin-neuraminidase and fusion glycoproteins of recombinant parainfluenza virus type 3 (PIV3) with their counterparts from PIV2 yields attenuated PIV2 vaccine candidates. *J. Virol.* 74(14), 6448–6458.

Tao, T., Skiadopoulos, M. H., Durbin, A. P., Davoodi, F., Collins, P. L., and Murphy, B. R. (1999). A live attenuated chimeric recombinant parainfluenza virus (PIV) encoding the internal proteins of PIV type 3 and the surface glycoproteins of PIV type 1 induces complete resistance to PIV1 challenge and partial resistance to PIV3 challenge. *Vaccine* 17(9–10), 1100–1108.

Teng, M. N., and Collins, P. L. (1999). Altered growth characteristics of recombinant respiratory syncytial viruses which do not produce NS2 protein. *J. Virol.* 73(1), 466–473.

Teng, M. N., Whitehead, S. S., Bermingham, A., St Claire, M., Elkins, W. R., Murphy, B. R., and Collins, P. L. (2000). Recombinant respiratory syncytial virus that does not express the NS1 or M2-2 protein is highly attenuated and immunogenic in chimpanzees. *J. Virol.* 74(19), 9317–9321.

Whitehead, S. S., Firestone, C. Y., Karron, R. A., Crowe, J., Jr., Elkins, W. R., Collins, P. L., and Murphy, B. R. (1999a). Addition of a missense mutation present in the L gene of respiratory syncytial virus (RSV) cpts530/1030 to RSV vaccine candidate cpts248/404 increases its attenuation and temperature sensitivity. *J. Virol.* 73(2), 871–877.

Whitehead, S. S., Hill, M. G., Firestone, C. Y., St Claire, M., Elkins, W. R., Murphy, B. R., and Collins, P. L. (1999b). Replacement of the F and G proteins of respiratory syncytial virus (RSV) subgroup A with those of subgroup B generates chimeric live attenuated RSV subgroup B vaccine candidates. *J. Virol.* 73(12), 9773–9780.

Wright, P. F., Karron, R. A., Belshe, R. B., Thompson, J., Crowe, Jr., J. E., Boyce, T. G., Halburnt, L. L., Reed, G. W., Whitehead, S. S., Anderson, E. L., Wittek, A. E., Casey, R., Eichelberger, M., Thumar, B., Randolph, V. B., Udem, S. A., Chanock, R. M., and Murphy, B. R. (2000). Evaluation of a live, cold-passaged, temperature-sensitive, respiratory syncytial virus vaccine candidate in infancy. *J. Infect. Dis.* 182, 1331–1342.

Wright, P. F., Shinozaki, T., Fleet, W., Sell, S. H., Thompson, J., and Karzon, D. T. (1976). Evaluation of a live, attenuated respiratory syncytial virus vaccine in infants. *J. Pediatr.* 88(6), 931–936.

Exhibit 2

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24 October 2003

Dr. Christian Abderhalden
Pennie & Edmonds, L.L.P.
1155 Avenue of the Americas
New York, NY 10036

Dear Dr. Abderhalden:

The publication date (i.e., mailing date) of the February 1994 issue of the *Journal of Virology* was 10 January 1994.

Sincerely,

Linda M. Illig
Director, Journals

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